

A novel spalt gene expressed in branchial arches affects the ability of cranial neural crest cells to populate sensory ganglia

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Cranial neural crest cells differentiate into diverse derivatives including neurons and glia of the cranial ganglia, and cartilage and bone of the facial skeleton. Here, we explore the function of a novel transcription factor of the spalt family that might be involved in early cell-lineage decisions of the avian neural crest. The chicken spalt4 gene (csal4) is expressed in the neural tube, migrating neural crest, branchial arches and, transiently, in the cranial ectoderm. Later, it is expressed in the mesectodermal, but not neuronal or glial, derivatives of midbrain and hindbrain neural crest. After over-expression by electroporation into the cranial neural tube and neural crest, we observed a marked redistribution of electroporated neural crest cells in the vicinity of the trigeminal ganglion. In control-electroporated embryos, numerous, labeled neural crest cells (~80% of the population) entered the ganglion, many of which differentiated into neurons. By contrast, few (~30% of the population) spalt-electroporated neural crest cells entered the trigeminal ganglion. Instead, they localized in the mesenchyme around the ganglionic periphery or continued further ventrally to the branchial arches. Interestingly, little or no expression of differentiation markers for neurons or other cell types was observed in spalt-electroporated neural crest cells.

Keywords: Spalt, neural crest, branchial arches, trigeminal ganglia

INTRODUCTION

The neural crest is a migratory cell type that originates from the dorsal neural tube and emigrates shortly after its closure. At cranial levels, neural crest cells migrate beneath the ectoderm and populate many ganglia of the PNS, differentiating into the glia and some of the neurons within these ganglia. In addition to neural derivatives, the neural crest cells that invade the head and the branchial arches give rise to most of the cartilage, bone and connective tissue of the face. How cells within the cranial neural crest population home to the proper structures and, subsequently, make cell-lineage decisions to differentiate into cell types as divergent as cartilage, neurons and glia remain unknown.

In the trunk region, several signaling pathways and transcription factors have been implicated in cell fate decisions of neural crest. For example, in dorsal root ganglia, Notch signaling appears to influence the decision to assume neuronal versus glial fates (Morrison *et al.*, 2000; Wakamatsu *et al.*, 2000). In the autonomic nervous system, bone morphogenetic protein (BMP) biases neural crest precursors toward a neural fate whereas glial growth factor/neuregulin drives cells into the glial lineage (Shah *et al.*, 1994; Shah *et al.*, 1996; Shah *et al.*, 1997). At the transcriptional level, null mutations in *Mash-1* lead to selective loss of sympathetic ganglion (Guillemot *et al.*, 1993) whereas overexpression of neurogenin biases neural crest cells toward the sensory lineage (Perez *et al.*, 1999). Transcription factors that affect cell localization and lineage decisions in cranial neural crest cells have yet to be described.

The spalt genes are a family of evolutionarily conserved zinc finger-containing transcription factors, mutations of which lead to homeotic transformations in *Drosophila* (Kühnlein

et al., 1994). Loss of *Drosophila spalt* transforms prothorax into head and abdominal segments into tail (Jürgens, 1988). Selective deletion of *Drosophila spalt* in the embryonic PNS causes a cell-fate switch in neuronal precursors and defects in migration (Rusten *et al.*, 2001; Elstob *et al.*, 2001). In the thorax, *spalt* and *spalt-related* are involved in positioning of specific proneural clusters (de Celis *et al.*, 1999). In addition, *spalt* is required for the directed migration of dorsal trunk cells in the trachea (Kühnlein and Schuh, 1996). Thus, this gene family appears to play important roles in cell migration and cell-fate decisions.

Here, we describe a novel chicken transcription factor of the spalt family that might be involved in specification of avian cranial neural crest cell lineages. We have isolated a chicken spalt gene, *csal4*, which is expressed in the neural tube, migrating neural crest and branchial arches. Both the sequence and expression pattern most closely resemble that of *Xenopus xsal-3* (Onuma *et al.*, 1999) and mouse *Sall4* (Kolhase *et al.*, 2002). Overexpression of this spalt cDNA in migrating cranial neural crest cells causes a redistribution of neural crest derivatives within the trigeminal ganglia to its periphery; furthermore, these *spalt*-expressing cells fail to express detectable differentiation markers of neurons or other cell types examined. This suggests that spalt can affect the localization of cranial neural crest cells, which, in turn, might influence the ability to form sensory neurons.

OBJECTIVE

We hypothesized that *csal4* might influence cranial neural crest migration and/or lineage decisions, based on its expression in

neural crest-derived branchial arches but not sensory ganglia and its known function in cell fate and migration decisions in *Drosophila*. To test this idea, we overexpressed the transcription factor in migrating neural crest cells, which resulted in altered localization of transfected cells to the periphery but not the core of the trigeminal ganglion where neurons form.

METHODS

Isolation of *csal4*

csal4 was isolated using RT-PCR from RNA derived from stage-8 embryos using the oligonucleotides OLF UP (5'-ATGTGGAGAACTAGTTTG-3') and OLF DN (5'-CCACAGATGATGAAGGCA-3') and the Access RT-PCR system (Promega). DNA fragments of 450 bp were isolated and cloned into pGEM-T (Promega). Two independent clones were identified and used to screen a stage 10 chicken-embryo cDNA library in HYBRIDZAP 2.1 (Stratagene). Several cDNA clones were isolated, including one that contained a 6-kb insert. Sequence analysis of the longest clone showed that it contained all but the most 5' sequence of the coding region. The 5' coding sequence was amplified from the stage 10 cDNA library. The deduced amino acid sequence of the entire coding region was compared to that of other spalt genes using the ClustalW program (Thompson *et al.*, 1994).

In situ hybridization

The complete *csal4* cDNA and several fragments were used as templates to prepare antisense RNA probes. In situ hybridization was performed as described (Wilkinson, 1992). The embryos were staged according to the criteria of Hamburger and Hamilton (Hamburger and Hamilton, 1951), fixed and embedded in gelatin and sectioned in a cryostat at 20 μ m. Gelatin was removed from the sections by soaking for 5 minutes in PBS at 42°C. Some of these sections were used for immunohistochemistry.

Overexpression of *csal4* by electroporation

The coding region of *csal4* was cloned into the pCIG vector (Megason and McMahon, 2002) downstream of a chicken β -actin promoter and upstream of enhanced green fluorescent protein (EGFP) under the control of an internal ribosomal entry site (IRES) sequence. The plasmid in 10 mM tris was diluted 1:1 with Ringers solution and injected into the lumen of the neural tube of embryos with 3–6 somites. Two pulses of 25 volts and 50 msec duration were delivered through two platinum electrodes, 4 mm apart, placed on top of the vitelline membrane on either side of the embryo (Itasaki *et al.*, 1999). The embryos were placed at 38°C for up to 48 hours of further development. Embryos were collected, fixed in 4% paraformaldehyde overnight at 4°C, embedded in gelatin and sectioned on a cryostat at 10 μ m. Sections were then stained with antibodies that recognize green fluorescent protein (GFP) (Abcam), and HNK-1 and TUJ1 (Covance). Anti-GFP antibodies were used to identify cells containing the pCIG plasmid and HNK-1 to identify neural crest cells. TuJ1 (against β 3-tubulin) immunostaining were done after electroporation to identify differentiated neurons. They were visualized by Alexa 568-conjugated donkey anti-mouse IgG or Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes).

RESULTS

Isolation of a chick *spalt* homologue *csal4*

Using an RT-PCR approach followed by library screening, we isolated a full length cDNA clone for a novel chicken spalt gene, *csal4* (GenBank accession number AY342354). The coding region contains three sets of zinc fingers and a single zinc-finger domain at the amino terminus, a glutamine rich region, and the 8 amino acid motif, referred to as the SAL-box, that is common to all spalt genes (Fig. 1A). Comparison with full length sequences of other spalt genes reveals that it is most closely related to *Xsal3* (61% identity at the amino acid level) and human *SALL4* (57% identity at the amino acid level). A dendrogram of the ClustalW data places these three genes in one of four separate spalt groups (Fig. 1B). However, the degree of sequence conservation between *csal4*, *Xsal3* and *SALL4* is less than that the relatedness observed within other groups. For example, human *SALL1* is 86% identical to chicken *csal1* and human *SALL3* is 71% identical to chicken *csal3*. No more closely related genes were uncovered by searches of genomic and EST databases. These results indicate that *csal4*, *Xsal3* and *SALL4* are homologues, but we cannot rule out the possibility that undiscovered discovered gene might be more related.

Expression pattern of *csal4* in chicken embryos

We examined the pattern of *csal4* expression by in situ hybridization of chicken embryos from the early neurula (E1) stage to the time of gangliogenesis (E3). As the neural folds are beginning to elevate (3-somite stage), *csal4* expression is found throughout the head ectoderm and the neural plate. A few hours later, as the neural tube begins to close at the level of the forebrain/midbrain, *csal4* expression is downregulated from the forming neural tube, but remains expressed in more caudal regions of the open neural plate (not shown).

By the 10-somite stage, there is strong staining in the lateral ectoderm and caudal dorsal neural tube. The otic placode, lateral ectoderm, pharyngeal endoderm, early somites and the notochord express *csal4*. Expression is also seen in migrating neural crest (Fig. 2A,B) and is particularly notable in cells emigrating from the hindbrain of stage-13 embryos (Fig. 2D,E). To verify that these *csal4*-positive cells are neural crest cells, embryos were sectioned following whole mount in situ hybridization and the sections stained with the HNK-1 antibody, which is a marker for early migrating neural crest cells (Bronner-Fraser, 1986). The *csal4* and HNK-1 expression patterns overlapped, which indicates that *csal4* is expressed in this migratory population (Fig. 2C,F).

The mesoderm in the branchial arches strongly expresses *csal4*, with highest levels in branchial arch 1 (Fig. 3A,D). Because the migrating neural crest marker, HNK-1, is downregulated as neural crest cells enter the branchial arches, we used a pan-Dlx antibody that recognizes all Dlx proteins (Panganiban *et al.*, 1997) as a marker for neural crest cells within this location (Panganiban and Rubenstein, 2002). We found extensive overlap of *csal4* RNA and Dlx protein at stages 14 and 15 in the branchial arches (compare Fig. 3B,C and Fig. 3E,F). However, the overlap was not absolute because there were areas that either expressed *csal4* only or Dlx proteins only. For example, during neural crest migration, *csal4* was expressed primarily in the ventral portion of the arches

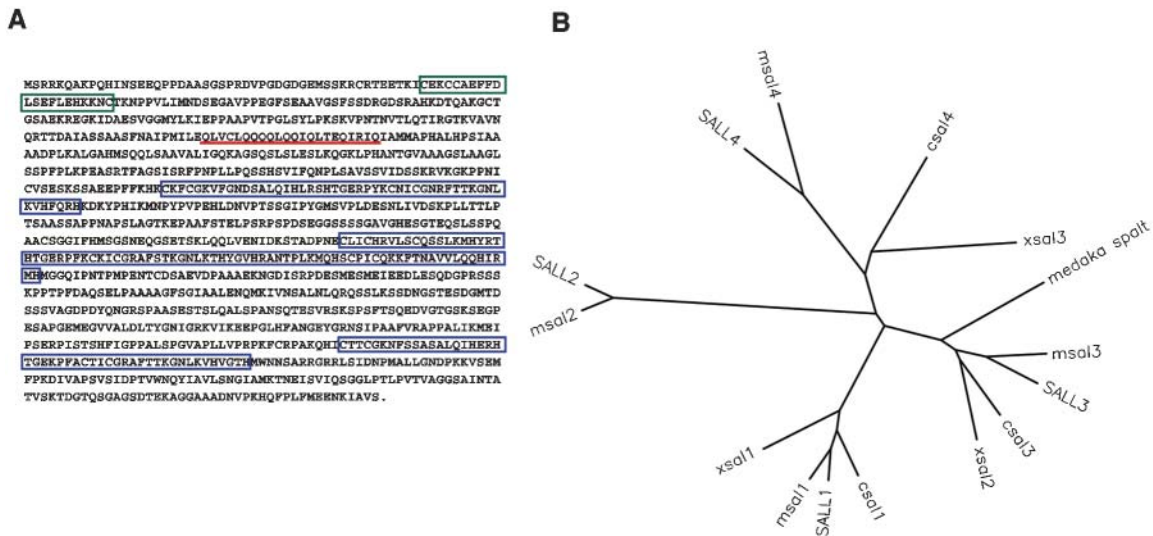


Fig. 1. Sequence analysis of *csal4*. (A) Amino-acid sequence of *csal4*. The nucleotide sequence has been assigned the GenBank accession number AY342354. The amino terminal CCHC zinc-finger region is boxed in green, the three C2H2 zinc-finger regions are boxed in blue and the glutamine-rich domain is underlined in red. (B) Dendrogram of ClustalW analysis of vertebrate spalt genes. There are four paralog groups of spalt. *csal4* is most closely related to *Xsal3* and human *SALL4*. The proteins (and their accession numbers) used in this analysis: human *SALL1* (Q9N5C2); human *SALL2* (Q9Y467); human *SALL3* (Q9BXA9); human *SALL4* (Q9UJQ4); mouse *msal1* (Q9ER74); mouse *msal2* (NP_056587); mouse *msal3* (XP_129051); mouse *msal4* (BAC33598); chicken *csal1* (AAG13011); chicken *csal3* (AAK38370); *Xenopus Xsal1* (AAG45108); *Xenopus Xsal2* (T30341); *Xenopus Xsal3* (JC7116); and medaka spalt (AAB51127). For clarity, the mouse genes are labeled to be consistent with the naming of the human genes.

whereas *Dlx* transcripts were also expressed in the dorsal portion (Fig. 3B,C). At later stages, *csal4* appeared to be expressed more medially than *Dlx* (Fig. 3E,F). This indicates that *csal4* is expressed in some, but not all, neural crest cells in the branchial arches.

By stage 15, the ectoderm no longer expresses *csal4* but expression continues in the otic vesicle (Fig. 3D). In the neural tube, *csal4* transcripts are observed in the forebrain, midbrain and hindbrain but are missing at the boundaries between these brain regions. At spinal cord levels, *csal4* is first expressed throughout the neural tube except for the most dorsal region; later *csal4* becomes restricted to a band of cells in the dorsal half of the spinal cord with the exception of the dorsal-most (presumptive roof plate) cells. However, *csal4* expression was undetectable in the trigeminal ganglia, which is made up of neural crest as well as placode cells (Fig. 3D).

Expression of *csal4* in the branchial arches remains strong until stage 25, the last stage examined. Collectively, these results indicate that *csal4* is expressed primarily in the branchial arch neural crest but is absent from the neuronal neural crest-derived population that emerges from the same axial level.

In addition to neural and neural crest derivatives, *csal4* transcripts are also found in the nephric ducts, notochord, sclerotomal portion of the trunk somites, pharyngeal endoderm and very strongly in the tail (data not shown). Staining was detected in the distal limb buds with faint staining in the somatic mesoderm at the level of the future wing bud (data not shown).

Overexpression of *csal4* alters localization of neural crest cells

We tested the possible function of *csal4* by overexpressing it in the forming nervous system using electroporation. The coding region of *csal4* was cloned into the pCIG expression vector that

coexpresses EGFP through an IRES sequence. This plasmid was injected into the lumen of the midbrain and hindbrain prior to neural crest migration and, subsequently, electroporated into the neural tube (Fig. 4A). This allows for transfection of the both neural tube and premigratory neural crest cells. At 1 day after electroporation, at a time when neural crest cells are at late stages of migration in the cranial region, no differences were noted between *csal4* and control-transfected cells, which indicates that there was no obvious effect on their early migratory behavior (data not shown). However, differences in the distribution pattern of electroporated experimental versus control neural crest cells were noted in embryos examined 48 hours after electroporation, by which time neural crest-derived cranial ganglia have formed (Fig. 4B). We focused our studies particularly on the trigeminal ganglion, a cranial sensory ganglion that receives a contribution from neural crest cells derived from the midbrain and rostral hindbrain.

Overexpression of *csal4* affected localization of neural crest cells within the trigeminal ganglion. Normally, neural crest cells contribute both neurons and glia of the trigeminal ganglia, with crest-derived neurons distributed throughout the proximal portion of the ganglion and non-neural/glia cells found both in the proximal and distal portions (Noden, 1980). Consistent with this, in control transfected embryos, GFP-labeled neural crest cells were distributed throughout the trigeminal ganglion in a relatively uniform pattern (Fig. 4E,F). By contrast, the bulk of the *csal4* expressing/GFP-labeled neural crest cells localized to the periphery of the ganglia and in the mesoderm adjacent to the ganglia (Fig. 4C,D). Only a few *csal4*-transfected cells were observed in the interior of the ganglion (Fig. 4C,D). In contrast to the trigeminal region, no obvious differences were noted in the distribution of electroporated neural crest cells in the branchial arch mesoderm of control versus *csal4*-transfected embryos (data not shown).

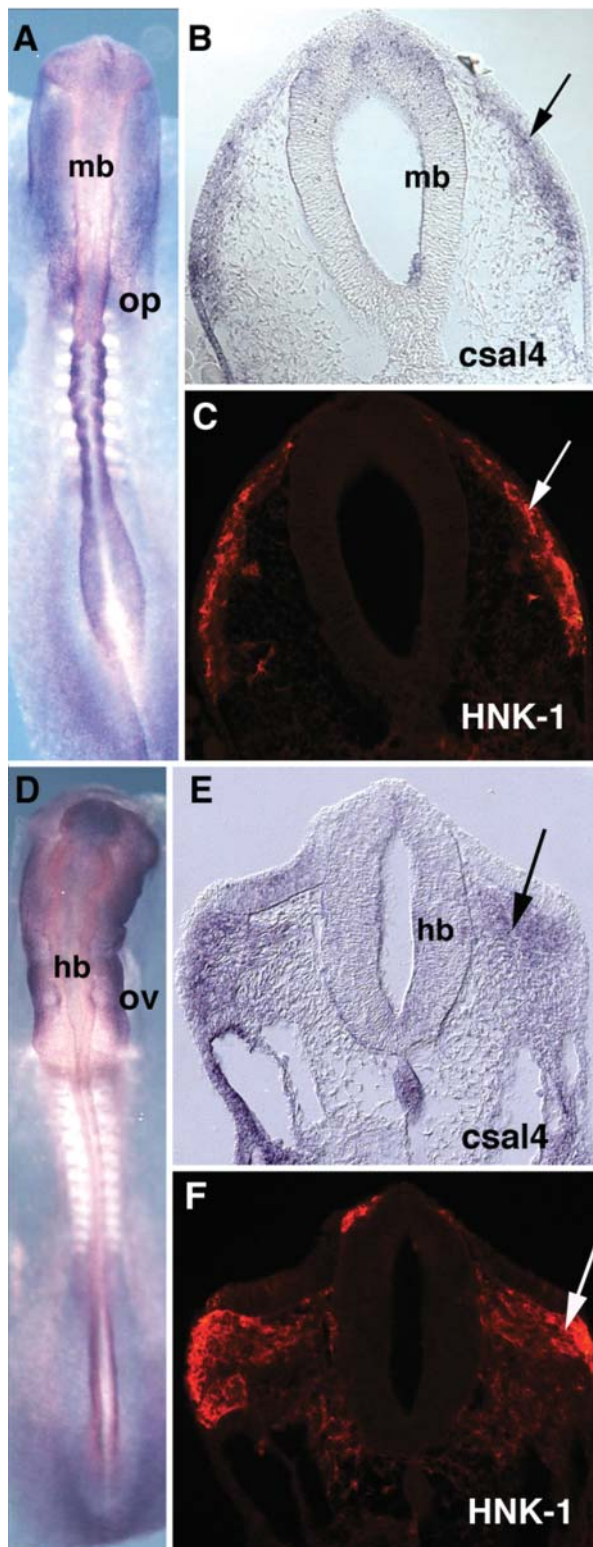


Fig. 2. Migrating neural crest cells express *csal4*. (A) Whole-mount in situ hybridization of a stage-10-chicken embryo with a *csal4* RNA probe. (B) Section through the midbrain of the embryo in (A). The arrow points to the migrating neural crest. (C) The same section as B, stained with HNK1 antibody. The arrow points to the migrating neural crest cells. The HNK1 antibody colocalizes with *csal4* expression. (D) Whole-mount in situ hybridization of a stage-13-chicken embryo with *csal4* probe. (E) Section of the embryo in D. The neural crest migrating from the hindbrain expresses *csal4* (arrow). (F) Same section as E, stained with HNK1 antibody. Arrows point to the migrating neural crest. At this level, *csal4* expression overlaps with HNK1. Abbreviations: hb, hindbrain; mb, midbrain; op, otic placode; ov, otic vesicle.

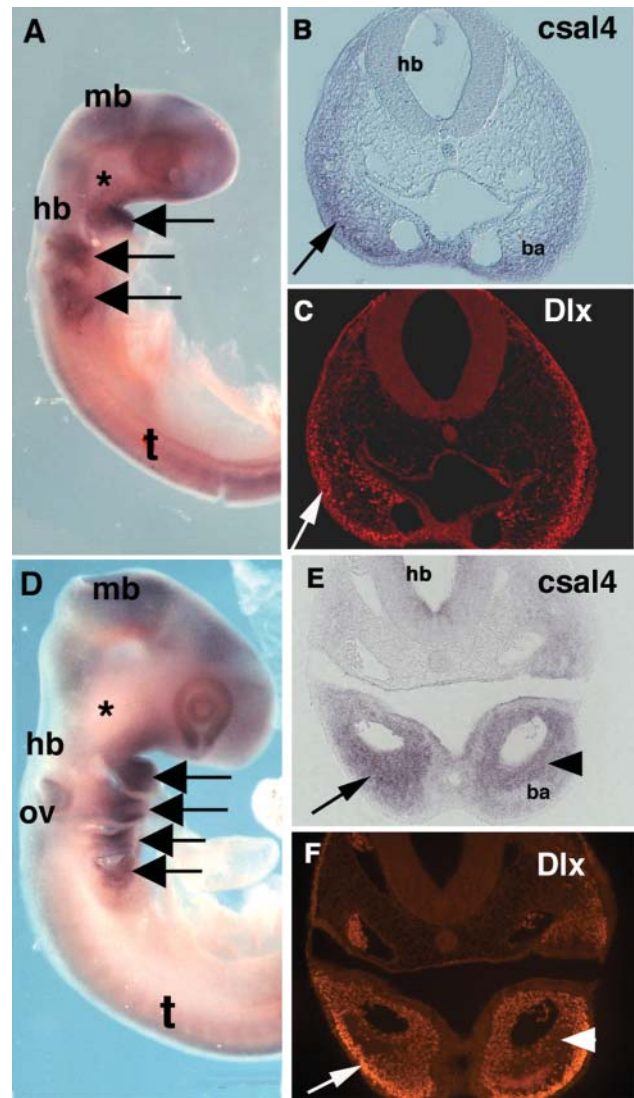


Fig. 3. Neural crest cells in the branchial arches express *csal4*. (A) Whole-mount in situ hybridization of stage-14-chicken embryo with *csal4* probe. A strong signal can be seen in the branchial arches (arrows) but there is no detectable signal at site of the trigeminal ganglia (asterisk). (B) Section through the region of the hindbrain of the embryo in A. Neural crest in the branchial arch (arrow) expresses *csal4*. (C) Same section as in B, stained with pan-Dlx antibody. Although not all Dlx-expressing cells express *csal4*, there is an overlapping population that expresses both (arrows). (D) Whole mount in situ hybridization of a stage-15-chicken embryo with *csal4* probe. All the branchial arches (arrows) but not the trigeminal ganglia (asterisk) express *csal4* strongly. (E) Section through the embryo in D showing extensive expression of *csal4* throughout the branchial arch (arrow) with highest levels in the core (arrowhead). (F) Section in E stained with pan-Dlx antibody. Although there is an overlapping population that expresses both *csal4* and Dlx protein, the highest level of Dlx expression (arrow) is distal to the region in which *csal4* expression is highest (arrowhead). Abbreviations: ba, branchial arch; hb, hindbrain; mb, midbrain; ov, otic vesicle; t, trunk.

The number of GFP-labeled cells in the mesoderm adjacent to the trigeminal ganglion and at the periphery of the trigeminal ganglia as well as those inside the ganglion were counted after electroporation of control or *csal4* plasmids. A total of 1053 GFP-positive cells were counted in six *csal4*-GFP transfected embryos and compared with 1666 cells in six control transfected embryos. We noted a significant difference between the percentage of electroporated cells that localized to the periphery of the ganglion versus total number of

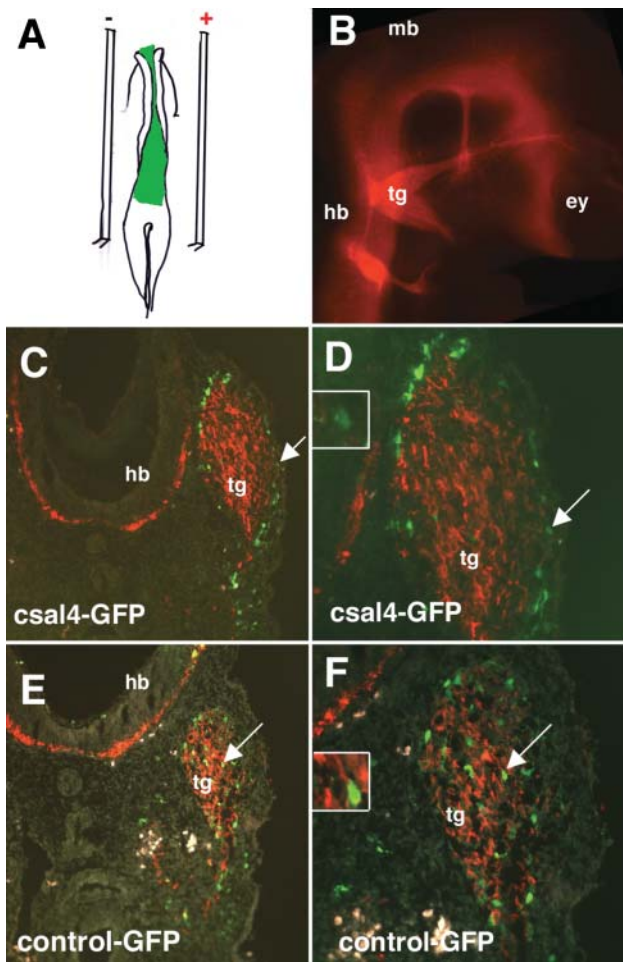


Fig. 4. Misexpression of *csal4* alters the localization of transfected neural crest cells in the vicinity of the trigeminal ganglia. (A) Schematic diagram illustrating the strategy for overexpressing *csal4*. Either *csal4* or control DNA solution, visualized with fast green, was injected in the lumen of the neural tube and the open neural plate (green). The anode and cathode electrodes are placed on top of the vitelline on either side of the embryo and two 50 msec pulses of 25 mv delivered. Embryos were incubated for an additional 48 hours. (B) Whole-mount immunofluorescence at the level of the head. TUJ1 antibody was used to stain the neurons of a stage-19 embryo to show the location of the cranial ganglia. (C) A transverse section at the level of the trigeminal ganglia through an embryo transfected with pCIG-sal expressing both *csal4* and GFP and stained with antibodies to GFP (green) and TUJ1 (red). A large fraction of the GFP-expressing cells (green, arrow) are localized on the periphery of the TUJ1-positive ganglia (red). (D) Higher magnification of C, showing that GFP-positive cells are outside the TUJ1-positive ganglia (see arrow). Inset is a detail of the cell marked by the arrow showing little to no detectable cytoplasmic TUJ1. (E) A transverse section at the level of the trigeminal ganglia through a control embryo transfected with pCIG expressing only GFP and stained with antibodies to GFP (green) and TUJ1 (red). Most of the GFP-positive cells are localized uniformly throughout the TUJ1-expressing ganglia (see arrow). (F) Higher magnification of E showing GFP-positive cells throughout the ganglia (arrow). Inset is a detail of the cell marked by the arrow showing cytoplasmic TUJ1 immunoreactivity in a cell with a GFP-positive nucleus. Abbreviations: ey, eye; hb, hindbrain; mb, midbrain; tg, trigeminal ganglia.

GFP-positive cells in the region of the ganglia. In *csal4*-electroporated embryos, 79% of the GFP-positive cells were localized to the periphery whereas in control electroporated embryos only 28% of GFP-positive cells were localized to the periphery ($P < 0.001$). These results demonstrate that overexpression of *csal4* alters the localization of neural crest cells.

Neural crest cells overexpressing *csal4* fail to express differentiation markers for neurons or other cell types

To assess whether the types of neural crest derivatives were altered after overexpression of *csal4*, sections through control and *csal4*-transfected embryos were stained with the neural marker TUJ1 that recognizes $\beta 3$ tubulin. In control electroporated embryos, numerous GFP-positive cells were present in the trigeminal ganglion. Many of these GFP-expressing cells were also positive for TUJ1 (Fig. 4E,F). By contrast, no TUJ1-positive cells were observed in GFP/*csal4*-expressing cells. Although the majority of these cells were found in the periphery where neurons do not typically differentiate, none of the labeled cells (28%) found within the ganglion expressed Tuji (Fig. 4C,D). In addition, the *csal4*-expressing cells within or at the periphery of the ganglion did not stain with the Hu antibody, another pan-neuronal marker, nor did they express the transcription factor, neurogenin-1 (data not shown). These results indicate that *csal4*-electroporated cells do not express detectable markers of neuronal differentiation.

We next looked for other signs of differentiation in the *spalt*-expressing population. The *csal4*-GFP cells on the periphery of the ganglion or in the mesoderm did not express smooth muscle actin, indicating that they had not differentiated into smooth muscle (data not shown). Nor did they stain with Dlx 3 antibody, suggesting that they had not assumed properties of branchial arch neural crest (data not shown). At the time of our analysis, glial cells have not yet differentiated so we were unable to assess glial fate in these embryos. Given the lack of differentiation markers in the electroporated cell, an intriguing possibility is that *spalt4* maintains cells in an undifferentiated state.

CONCLUSIONS

- Chicken *spalt4* is expressed in early migrating cranial neural crest cells; later, it is maintained in the population within the branchial arches but absent in the trigeminal ganglion.
- Over-expression of *csal4* in the cranial neural crest alters their pattern of localization within the trigeminal ganglia but does not affect migration within the branchial arches; *spalt4*-expressing neural crest cells localize primarily in the periphery rather than in the core of the ganglia.
- Neural crest cells over-expressing *csal4* do not express detectable neuronal or other differentiation markers.

DISCUSSION

In this study, we have isolated a novel chicken *spalt*-family member that biases cell localization and might alter fate decisions in the cranial neural crest. Based on sequence similarity and expression pattern, we surmise that *csal4*, *Xsal3* and *SALL4* are likely to be homologues. Although the degree of sequence conservation between *csal4*, *Xsal3* and *SALL4* is less than that the relatedness observed within other groups, *csal4*, *Xsal3* (Onuma *et al.*, 1999) and mouse *Sall 4* (Kollhase *et al.*, 2002) have similar expression patterns in the neural tube and the neural crest, as well as the limbs and other tissues, consistent with these being true homologues.

In the early embryo, strong expression of *csal4* was noted in both the neural plate and the ectoderm as early as stage 7. At subsequent stages, expression in the neural tube is downregulated but expression in the ectoderm is maintained in some placode regions. Neural tube expression reappears later at variable levels depending upon age and rostrocaudal location. In the brain, *csal4* expression declines after neural tube closure, but is re-expressed by stage 13, when it is present in the forebrain, midbrain and hindbrain with the exception of the borders between them. This is in contrast with the expression of another chicken spalt gene, *csal3*, which is expressed only in the border regions (Farrell *et al.*, 2001). Thus, the two spalt genes have complementary, non-overlapping patterns in the head. At the level of the trunk, *csal4* is expressed first throughout the neural plate. Later, expression in the most dorsal cells is reduced and afterwards the expression in the ventral neural tube becomes downregulated, which leaves a stripe of *csal4* expression in the dorsal half of the spinal cord.

Neural crest cells migrate from the midbrain and the hindbrain to form the cartilage, bone and connective tissue of the face as well as the glia and many neurons of the cranial ganglia. We find expression of *csal4* in the migrating neural crest of the hindbrain as early as stage 10. However, at later stages of development, only a subset of neural crest cells express *csal4*, as seen by comparing the in situ pattern of *csal4* with the antibody pattern of the neural crest marker HNK1. Once the neural crest cells migrate into the branchial arches, *csal4* is detected in the arches in many cells that co-express *Dlx*, although the overlap is not complete. The co-expression of *Dlx* and in the branchial arches presents an interesting parallel with *Drosophila* where *distalless* (the *Drosophila* *Dlx* homolog) has been shown to induce *spalt* in the antenna (Dong *et al.*, 2000).

The expression of *csal4* in the branchial arches but absence from the cranial ganglia raises the interesting possibility that *csal4* might bias cell fate decisions toward cartilage and bone lineages at the expense of neurons and/or glia. To test for possible effects of *csal4* on neural crest cells, we over-expressed it in migrating neural crest cells of the developing chicken embryo via electroporation. The results show that excess spalt affects the localization of neural crest cells within the trigeminal ganglion. The cranial ganglia of embryos that over-express spalt contain significantly fewer GFP-positive cells than in control embryos. Most of the labeled cells failed to incorporate into the core of the ganglia itself and were found predominantly either at its edges or in the adjacent mesoderm.

Given that most of the *csal4*-electroporated cells localized at the periphery of the trigeminal ganglia whereas they were uniformly distributed throughout the ganglia in controls, an intriguing possibility is that these alterations in cell localization might be due to differences in cell adhesion as a consequence of spalt overexpression. Thus, the primary function of spalt could be to affect cell–cell adhesion, which, in turn, affects cell localization. If this is the case, it is possible that its influences on cell fate are a secondary consequence of the site of localization.

A second, but not mutually exclusive, possibility is that *csal4* might be involved in the cell fate decisions that determine whether a neural crest differentiates into branchial arch cartilage or forms neuronal derivatives in the neural crest-derived ganglia. The *csal4*-electroporated cells failed to express markers of neuronal differentiation, as assayed by TUJ1 staining. Even after 3 days (stage 23), *csal4*-expressing cells in the periphery failed to express the neuronal marker Hu. These

results indicate that downregulation of *csal4* might be necessary for proper localization and/or neuronal differentiation within the ganglion.

If overexpression of *csal4* alters the distribution and, consequently, the differentiation of electroporated neural crest cells, what is the fate of these cells? One possibility is that many of the electroporated cells switch from neuronal fate to a mesodermal or glial fate. However, we were unable to detect phenotypic markers of glia, smooth muscle or branchial arch neural crest (which express *Dlx* genes) in the electroporated cells at the times examined. Given that they fail to express known differentiation markers, it is possible that they remain in an undifferentiated state. If this is the case, *csal4* may keep cells in a 'stem-like' state.

Spalt was first isolated in *Drosophila* as a homeotic gene (Jürgens, 1988). It is expressed in two bands, one near the head and the other near the tail. Mutations in *spalt* cause prothorax structures to develop in the head and structures of the eighth abdominal segment to develop in the tail (Jürgens, 1988). *Spalt* is also involved during later cell fate decisions in *Drosophila* development, including eye (Mollereau *et al.*, 2001), trachea (Kühnlein and Schuh, 1996), and wing (de Celis *et al.*, 1996).

Regulation of spalt genes in vertebrates has been examined during medaka brain development (Köster *et al.*, 1997) and chicken limb development (Farrell and Münsterberg, 2000). These experiments show that spalt genes are under the control of a number of different signaling molecules, including sonic hedgehog, and members of the Wnt and FGF family of growth factors. Many of these growth factors are required both at early stages of neural crest development (Garcia-Castro *et al.*, 2002) and for proper patterning and differentiation of the branchial arches (LeDouarin and Kalcheim, 1999). One intriguing possibility is that they might be intimately interlinked with regulation of *csal4* in the migration of neural crest from the neural tube to the branchial arches.

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